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Pathways for Electron Tunneling in Cytochrome *c* Oxidase

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Warburg showed in 1929 that the photochemical action spectrum for CO dissociation from cytochrome *c* oxidase is that of a heme protein. Keilin had shown that cytochrome *a* does not react with oxygen, so he did not accept Warburg's view until 1939, when he discovered cytochrome a_3. The dinuclear cytochrome a_3-Cu_B unit was found by EPR in 1967, whereas the dinuclear nature of the Cu_A site was not universally accepted until oxidase crystal structures were published in 1995. There are negative redox interactions between cytochrome *a* and the other redox sites in the oxidase, so that the reduction potential of a particular site depends on the redox states of the other sites. Calculated electron-tunneling pathways for internal electron transfer in the oxidase indicate that the coupling-limited rates are 9×10^5 ($Cu_A \rightarrow a$) and 7×10^6 s^{-1} ($a \rightarrow a_3$); these calculations are in reasonable agreement with experimental rates, after corrections are made for driving force and reorganization energy. The best Cu_A-*a* pathway starts from the ligand His204 and not from the bridging sulfur of Cys196, and an efficient *a*-a_3 path involves the heme ligands His378 and His376 as well as the intervening Phe377 residue. All direct paths from Cu_A to a_3 are poor, indicating that direct $Cu_A \rightarrow a_3$ electron transfer is much slower than the $Cu_A \rightarrow a$ reaction. The pathways model suggests a means for gating the electron flow in redox-linked proton pumps.

KEY WORDS: Cytochrome oxidase; electron transfer; pathways method; proton pump.

INTRODUCTION

All redox-linked proton pumps are molecular machines, in which electron transfer from a donor to an acceptor is mechanistically coupled to the translocation of protons across a membrane. To effect coupling, the electron flow must be gated (DeVault, 1971). This is achieved by the transducer (pump) existing in two discrete conformational states, one being the input state for electrons from the donor and the other the output state to the acceptor. If the same states are also the input and output states for protons, a reaction scheme involving eight states is obtained, i.e., each conformational state can be in an oxidized or reduced form, unprotonated or protonated, respectively (Wikström *et al.*, 1981). It thus follows that in order to understand the pump mechanism, one must know the electron-transfer rate to and from the pump element in different states of the transducer. In this essay, we will analyze the principal internal electron-transfer reactions in one specific proton pump, cytochrome *c* oxidase, the terminal enzyme of the respiratory chain.

For most real proton pumps, the problem just presented is compounded by the fact that the tranducer contains not one, but many, potential pump elements. In Fig. 1, we present the redox centers of cytochrome oxidase. It is seen that there is a dinuclear Cu_A center, which has been shown to be the primary acceptor of electrons from cytochrome *c*, the initial electron donor (Brzezinski *et al.*, 1995). From Cu_A, the electron is transferred to cytochrome *a* and then to the cytochrome a_3-Cu_B center (see Winkler *et al.*, 1995), where the reduction of dioxygen to two molecules of water takes

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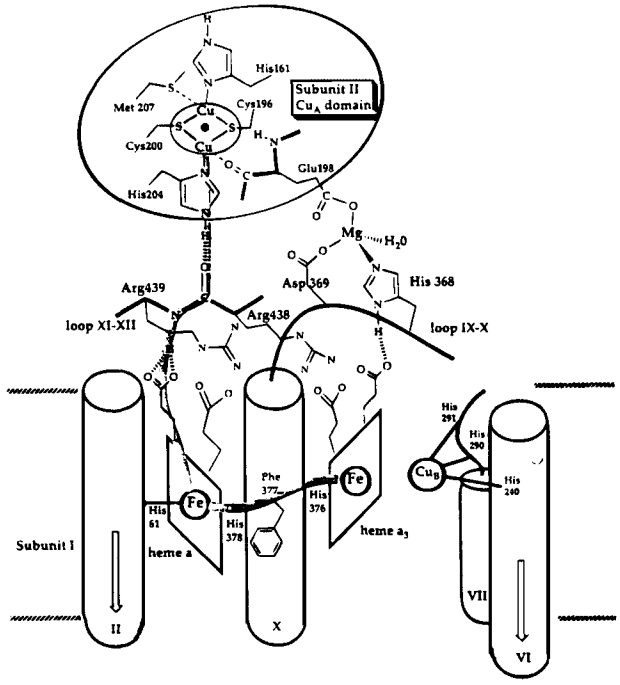

Fig. 1. Diagram showing linkages between subunits I and II of cytochrome oxidase based on the atomic coordinates of Iwata *et al.* (1995) (note that the amino acid numbers refer to the bovine enzyme). The oval at the top represents the soluble cupredoxin-like domain of subunit II. The Cu_2S_2 rhomb of Cu_A is shaded. Cylinders are transmembrane helices of subunit I (arrows indicate the direction of the polypeptide chain). The porphyrin rings of the hemes of cytochromes *a* and a_3 are drawn as squares with the propionate groups highlighted. Two loops, loop IX-X and loop XI-XII, that connect transmembrane helices in subunit I are also shown. Hydrogen bonds of a peptide unit in loop XI–XII form part of a good Cu_A-to-heme *a* electron-transfer pathway (shaded). A direct electron-transfer pathway, via Phe377, between heme *a* and heme a_3 is also evident (shaded).

place. Here we show that the rates of these internal electron transfers accord with semiclassical theory in which electronic couplings are estimated from an analysis of the tunneling pathways that connect the distant redox centers. The analysis will be preceded by a summary of the structures and redox properties of the centers. But first a brief discussion of the early history of the field is in order.

CYTOCHROME OXIDASE AS A HEME-COPPER OXIDASE

In a special issue devoted to heme-copper oxidases, it may be appropriate to summarize some of the initial work leading to the concept of such a group of enzymes, the earliest member of the group being cytochrome oxidase. The first clear evidence that cytochrome oxidase is a heme protein came in 1929, when Otto Warburg demonstrated that the photochemical action spectrum for the dissociation of the CO complex of the reduced "Atmungsferment," his name for cytochrome oxidase, is similar to that of hemoglobin (Warburg and Negelein, 1929). This was not accepted by the other great cytochrome oxidase pioneer, David Keilin, since his cytochrome *a* did not react with dioxygen. Instead, Keilin maintained that cytochrome oxidase is a copper protein (Keilin and Hartree, 1938). In 1939, however, Keilin discovered cytochrome a_3 and found that it behaved as Warburg's Atmungsferment (Keilin and Hartree, 1939).

The idea that cytochrome oxidase might contain copper was more or less forgotten until 1959, when Beinert reported the EPR spectrum of what we now know as Cu_A (Sands and Beinert, 1959). That this is a functional component was not immediately accepted. For example, at a conference in 1964, Chance maintained that its reduction is too slow to be part of turnover (see King *et al.*, 1965). At the same conference, Beinert reported that there are two intrinsic copper sites in cytochrome oxidase (Beinert, 1965), and a few years later he discovered the dinuclear nature of the cytochrome a_3-Cu_B center (van Gelder *et al.*, 1967).

ELECTRONIC STRUCTURES OF THE REDOX CENTERS

With the expression of soluble Cu_A domains from the COOH-terminal portion of subunit II of the oxidase (Lappalainen *et al.*, 1993; Slutter *et al.*, 1996) and with the determination of the crystal structure of two cytochrome oxidases (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995) as well as of an engineered Cu_A domain (Wilmanns *et al.*, 1995), it became established that the Cu_A center is a dinuclear copper complex. It consists of a planar ring formed by the two Cu ions and two bridging cysteine thiolate sulfur atoms; in addition, each Cu is coordinated to a terminal histidine as well as a weak axial ligand, as shown in Fig. 1. EPR (Karpefors *et al.*, 1996) and NMR (Bertini *et al.*, 1996) studies have clearly demonstrated that the unpaired electron in Cu_A is in an orbital (SOMO) that is equally distributed over the two halves of the center, with a high degree of delocalization onto the S atoms, whereas there is rather little spin density on the histidine N atoms.

Both cytochrome *a* and cytochrome a_3 contain a heme A coordinated in different ways in subunit I (Fig. 1), without any covalent attachment of the porphyrin to the protein. Cytochrome *a* has two axial His ligands provided by different helices, with the result that the heme is low-spin in both oxidation states. Cytochrome a_3 has one axial His ligand located on the opposite side of the helix, providing one of the cytochrome *a* ligands (Fig. 1). This results in a high-spin heme, which, however, cannot be detected by EPR in the oxidized protein, because it is antiferromagnetically coupled to Cu_B (van Gelder and Beinert, 1969; Aasa *et al.*, 1976), which is located 5.2 Å from the heme Fe. In partially reduced oxidase, a *g*6 signal appears from the high-spin heme in molecules with Cu_B reduced (Aasa *et al.*, 1976). This turns into a low-spin signal at high pH due to binding of an OH^- to Fe^{3+}.

REDUCTION POTENTIALS OF THE REDOX CENTERS

It has long been known that potentiometric titrations of cytochrome oxidase monitored by the electronic absorption of the hemes yield sigmoidal Nernst plots, corresponding to midpoint potentials of 380 and 230 mV, respectively (see Wikström *et al.*, 1981). Originally it was thought that these values represent the individual potentials of the two cytochromes, but it was later pointed out that the results are equally well explained in terms of a negative potential interaction between the hemes, i.e., the potential of one center depends on the redox state of the other (Malmström, 1973). This could explain the earlier puzzling observation that identical Nernst plots were obtained at 605 and 445 nm, despite the fact that the spectral contributions of the two cytochromes at these wavelengths are quite different. Thus, according to this analysis, both cytochrome *a* and a_3 constitute the high- as well as the low-potential heme.

A shortcoming in the model just presented is that it considers heme–heme interactions only and ignores the other redox sites. A later investigation demonstrated that cytochrome *a* displays a negative interaction with all three of the other redox sites and that there is also such an interaction between cytochrome a_3 and Cu_B (Blair *et al.*, 1986). These interactions will be considered when estimating the driving force in a subsequent analysis of the rates of internal electron-transfer steps.

RATES OF INTERNAL ELECTRON TRANSFER

Boelens *et al.* (1982) demonstrated that there is a backflow of electrons on photolysis of the CO compound of the half-reduced oxidase, and this approach has later been used by many investigators to study in detail the rates of internal electron-transfer steps and their temperature and pH dependences (see Ädelroth *et al.*, 1995, and references therein). In these experiments, a rate constant of 3×10^5 s^{-1} is observed for electron equilibration between cytochromes a_3 and *a*; the *a* to a_3 rate is 2×10^5 at a driving force of 40 meV. The rate of electron transfer from Cu_A to cytochrome *a* is 2×10^4 s^{-1}; when the a_3-Cu_B site is reduced, this rate constant is 1×10^4 s^{-1}.

Several authors have analyzed the results just recapitulated in terms of electron-transfer theory (Winkler *et al.*, 1995; Ramirez *et al.*, 1995). In particular, attempts have been made to estimate the reorganization energy, λ, associated with electron transfer (Brzezinski, 1996). For the $a \rightarrow a_3$, transfer, a value of 0.76 eV was found, which is in the range of typical λ values for protein electron transfer (Gray and Winkler, 1996). For $Cu_A \rightarrow a$, on the other hand, a λ as low as 0.3 eV was estimated. The fact that the $Cu_A \rightarrow a_3$ reaction is very slow, despite the similar Cu-to-Fe distance compared to that for the $Cu_A \rightarrow a$ electron transfer, was ascribed to a low driving force and a λ of 0.8 eV.

PATHWAY ANALYSIS

The two major internal electron-transfer reactions in cytochrome oxidase are $Cu_A \rightarrow a$ and $a \rightarrow a_3$-Cu_B. Since the Cu-to-Fe distance from Cu_A to cytochrome a_3 is only slightly longer than the corresponding Cu_A-to-*a* distance (21.1 Å compared to 19.5 Å), it has been suggested (Tsukihara *et al.*, 1995) that there is also rapid electron transfer from Cu_A to a_3. Consequently, we will examine this reaction as well. We have employed the pathways method pioneered by Beratan and Onuchic (Beratan *et al.*, 1992) as extended by one of us (Regan *et al.*, 1997) to identify electron-transfer tubes of specific covalent and hydrogen bonds that couple the redox centers of the oxidase.

For the $Cu_A \rightarrow a$ reaction, an electron-transfer pathway starting with a hydrogen bond between one of the ligand His residues (His204, bovine enzyme numbering) and a carbonyl group of a loop in subunit I has already been identified (Ramirez *et al.*, 1995).

This consists of 14 covalent bonds and 2 H bonds (an effective tunneling length of 25.2 Å), as seen in the wiring diagram in Fig. 2. The estimated activationless rate is 9×10^5 s^{-1}. At the driving force for this reaction (90 meV), the value of λ must be small to account for the experimentally observed rate (Brzezinski, 1996).

Williams *et al.* (1997) have suggested an alternative pathway, in which tunneling from the Cu_A site involves one of the bridging sulfurs (Cys196). In favor of such a pathway, the large amount of S(Cys) character in the SOMO of Cu_A, analogous to the situation in blue copper proteins, was quoted. The best pathway through Cys consists of 26 covalent bonds, 2 H bonds, and 1 through-space jump, which corresponds to a coupling-limited rate that is several orders of magnitude below the experimentally observed rate. A slightly more efficient Cys pathway uses a shortcut provided by a hydrogen bond between S(Cys) and a backbone amide group; it consists of 22 covalent bonds, 3 H bonds, and 1 through-space jump. The coupling of a path this length is still too weak to give the observed rate. We conclude that electron tunneling from Cu_A will go via His204, owing to its direct bond connectivity to the heme propionate of cytochrome *a*.

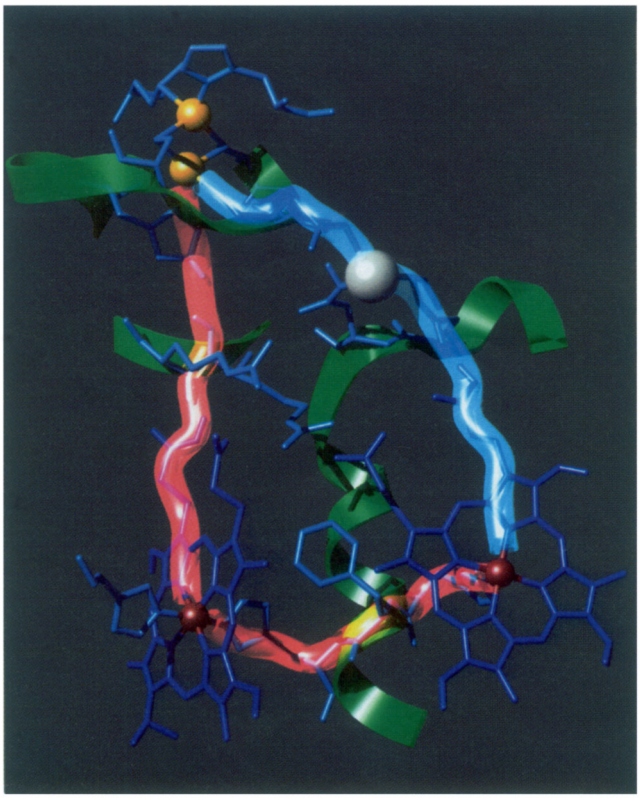

Fig. 2. Wiring diagram for the redox centers of cytochrome oxidase based on atomic coordinates from Tsukihara *et al.* (1995). On the left is the best path between Cu_A and heme *a* (red line), exploiting two hydrogen bonds (dashed lines). At the bottom the two irons are connected by a path from heme *a* to heme a_3 (magenta line). Two direct Cu_A-to-heme a_3 routes are shown for purposes of comparison (blue lines). In the middle of the figure is a route that does not use orbitals coupled to Mg^{2+}, and to the right and above is a route that does.

There are three almost equivalent pathways for the $a \rightarrow a_3$ reaction, all starting and ending at the Fe atoms of the hemes and going through the ligand histidines (His378 and His376, respectively). One goes through the single residue between the ligand histidines, Phe377, and comprises 16 covalent bonds (Fig. 2). Each of the other two pathways starts with the H bond between His378 and Val374 and then goes to Ala375. One continues directly to His376, and this path has 15 covalent bonds and one H bond. The third pathway goes from Ala375 via an H bond to Tyr372 and from there via another H bond to His 376, giving a total of 14 covalent and 3 H bonds. The calculated activationless rate for the $a \rightarrow a_3$ electron transfer is 7×10^6 s^{-1} (the tunneling length of the covalent path is 22.4 Å); with a reorganization energy of 0.44 eV, agreement with the experimental rate of 3×10^5 s^{-1} is obtained.

Dutton and co-workers insist (Moser *et al.*, 1996, 1997) that distances for electron transfer between heme groups in proteins should be edge-to-edge and not center-to-center, as used here. The edge-to-edge distance between the hemes of cytochrome *a* and cytochrome a_3 is only about 5 Å, and, from the Moser–Dutton rate/distance formula, the predicted activationless rate is 10^{11} s^{-1}. Assuming that the nuclear factor is within a reasonable range, it is not possible to reconcile this high coupling-limited rate with the experimental rate; furthermore, it is difficult to see how edge-edge transfer could be controlled, so this mechanism would seem to preclude electron gating in the proton pump.

For the hypothetical $Cu_A \rightarrow a_3$ electron transfer, there are two categories of pathways, those that exploit orbitals in the neighborhood of the Mg^{2+} ion and those that do not. An example of a path that does not approach Mg^{2+} can be seen in the middle of Fig. 2. It leaves Cu_A via His204 (like the best $Cu_A \rightarrow a$ path), and then traverses the length of the Arg438 residue, ultimately coupling through 22 orbitals. For the purpose of taking electrons away from Cu_A, this pathway cannot compete with the much shorter tunneling route to cytochrome *a*.

The paths that exploit Mg^{2+} (upper right in Fig. 2) leave Cu_A by either Cys196 or Glu198, traverse the bonds in the neighborhood of the Mg^{2+} ion, and finally use a long hydrogen bond connecting His368 (a Mg^{2+} ligand) to heme a_3. With an estimated tunneling length of 33.6 Å, the best route from Cu_A to a_3 via Mg^{2+} is still much longer than the $Cu_A \rightarrow a$ path. Indeed, with such weak coupling, the $Cu_A \rightarrow a_3$ rate would be only be $\sim 40\ s^{-1}$ (assuming a reorganization energy of 0.5 eV), which is more than two orders of magnitude smaller than the $Cu_A \rightarrow a$ rate. Thus, it is not surprising that the reaction is not observed.

CONCLUDING REMARKS

We have constructed a wiring diagram for the redox centers in cytochrome oxidase (Fig. 2). This can now be tested experimentally, since it is possible to introduce breaks in the defined electron-transfer paths by site-directed mutagenesis. Our findings also lend support to the thesis that long-range electronic couplings in proteins depend critically on the structure and composition of the intervening medium (Gray and Winkler, 1996; Winkler and Gray, 1997). The pathways model has, in addition, the advantage that it provides a means for gating the electron flow, a necessity in a redox-linked proton pump.

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